

AD _____

Award Number: DAMD17-01-1-0772

TITLE: Improving Blood Monitoring of Enzymes as Biomarkers of
Risk form Anticholinergic Pesticides and Chemical Warfare
Agents

PRINCIPAL INVESTIGATOR: Barry W. Wilson, Ph.D.

CONTRACTING ORGANIZATION: University of California
Davis, California 95616-8671

REPORT DATE: October 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20040206 095

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 2003	3. REPORT TYPE AND DATES COVERED Annual (24 Sep 2002 - 23 Sep 2003)	
4. TITLE AND SUBTITLE Improving Blood Monitoring of Enzymes as Biomarkers of Risk from Anticholinergic Pesticides and Chemical Warfare Agents			5. FUNDING NUMBERS DAMD17-01-1-0772	
6. AUTHOR(S) Barry W. Wilson, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California Davis, California 95616-8671 <i>E-Mail:</i> bwwilson@ucdavis.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Blood cholinesterase (ChE) biomarkers are an important way to monitor exposure to anticholinergic pesticides and chemical warfare (CW) agents and to establish whether some are at greater risk than others from exposure to them. Many clinical and research laboratories use the colorimetric Ellman assay based on the hydrolysis of acetylthiocholine. CHPPM (US Army Center For Health Promotion and Preventive Medicine) uses the Michel delta pH method to monitor more than 25,000 DoD personnel each year. Paired samples of RBCs assayed by CHPPM were sent to UC Davis to be assayed with acetylthiocholine by the Ellman method. Slopes of pH vs Ellman results for two sets of samples had poor correlations of r^2 of 0.64 and 0.14. Sample sets were assayed on day of receipt and the following to check on the timing of assay after shipping as a possible cause of the poor correlation. No effects were observed. Parallel work established conversion factors for clinical laboratories, assisting them in meeting California regulations and being approved for occupational ChE monitoring. Future work will involve Ellman and delta pH assays carried at the UC Davis laboratory to reduce variability as a next step in establishing a conversion between assays.				
14. SUBJECT TERMS Blood markers, chemical warfare agents, pesticides, cholinesterases				15. NUMBER OF PAGES 12
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	i
SF 298.....	ii
Table of Contents.....	iii
Introduction.....	1
Body.....	1
Key Research Accomplishments.....	4
Reportable Outcomes.....	4
Conclusions.....	5
References.....	5
Appendices.....	6

INTRODUCTION

There is a need for rapid, high throughput, reliable and transferable determinations of blood cholinesterase (ChE) levels to provide early warning of exposures due to the intensive use of pesticides such as organophosphate esters (OPs) and threats of chemical warfare agents. The colorimetric Ellman assay based on the hydrolysis of acetylthiocholine (Ellman, *et al.*, 1961) is used by many clinical and research laboratories. A slower delta pH method based on that of Michel (1949) is used to monitor more than 25,000 DOD personnel each year by the US Army Center For Health Promotion and Preventive Medicine (CHPPM). Although pH assays are reliable and have low variability, they are not readily adaptable for automation or field use.

One goal of this project is to establish a conversion factor between the pH and colorimetric assays applicable to monitoring studies and field tests. Another goal was to provide conversion factors for the portable Test-Mate kit manufactured by EQM, Inc. since studies have shown that the current model does not adequately adjust for temperature to be useful for field use. Plans agreed to by the manufacturer are to use a new model from the manufacturer with improved assay parameters. A bovine RBC AChE standard preparation we developed is used as a reference standard in assay comparisons.

Another issue is that of genetically sensitive individuals exposed to anticholinergic chemicals. Lowered butyrylcholinesterase (BChE), a scavenger of antiChE agents, may put individuals at increased risk from OP and CB agents (reviewed by Wilson, 1999) and a polymorphic form of paraoxonase (PON1), which destroys selected OPs, has been reported to be reduced in a cohort of veterans suffering from "Gulf War Syndrome" (Haley *et al.*, 1999). There is evidence that low levels of BChE and PON1 affect sensitivity to OP exposures of experimental animals (Shih *et al.*, 1998, Broomfield *et al.*, 1991). Following completion of the cholinesterase tasks, plans are to investigate the incidence of lowered BChE and PON1 in the blood samples.

BODY

Materials

All chemicals were purchased from Sigma Chemical Co.

Methods

Sample Handling

RBC samples are shipped overnight on cold packs by CHPPM personnel. Upon receipt at UC Davis, the temperature of the samples is checked (CHPPM protocol states samples will be <10°C during shipping). The samples are stored at 4°C and kept on ice during use. Ghost RBC samples are stored at -70°C, and shipped overnight on dry ice to CHPPM from UC Davis.

ChE Determinations

RBC samples received from CHPPM are diluted 1/50 in a lysis buffer (0.5% Triton X-100, 0.1 M sodium phosphate, pH 8) and their ChE activities measured using the colorimetric method of Ellman, *et al.* (1961), modified for use with an automatic microplate reader. Activity is determined in the presence and absence of 0.02 mM quinidine sulfate, a selective BChE inhibitor.

Ghost RBC Preparation

Bovine blood is centrifuged at 1000 x g and the plasma discarded. The RBCs are resuspended in isotonic buffer and washed twice. RBC ghosts are prepared by lysing the cells with hypertonic buffer. The membrane bound AChE is centrifuged at 100,000 x g and the pellet solubilized in buffer with Triton X-100 detergent. The solution is diluted and stored at -70°C (Arrieta *et al.*, 2003).

Task One.

Comparisons: The first task is to conduct a careful comparison of the Ellman assay performed under optimum conditions and the DOD pH assay to examine the variability and reliability of both assays, to establish baseline values and to generate conversion factors to enable comparisons between them and with other proposed or commercial assays. Last year five sets of RBC samples were sent from CHPPM to UC Davis. This year several more comparisons were undertaken (Figures 1-4). Although several sets showed good correlations and similar slopes between the laboratories, some did not (Tables 1 and 2). There was a concern about when the samples were assayed at the UC Davis laboratory and if a delay of one day affected the comparison. The latest sample sets were assayed on the day of receipt and the following day. There does not appear to be any effect on the correlation due to the timing of the assay (Table 2). Our next step is to do direct comparisons of the Ellman and pH assays in our own laboratory, eliminating possible variability introduced by shipping samples.

Table 1. Comparison of AChE Measurements at CHPPM and UC Davis

Sample Set	Slope	Correlation, r^2
1	0.047	0.74
2	0.045	0.79
3	0.028	0.25
4	0.048	0.80
5	0.025	0.34

Slopes are ratio of delta pH/Ellman AChE activities. Previously presented.

Table 2. Further Comparison of AChE Measurements at CHPPM and UC Davis

Sample Set	Slope	Correlation, r^2
6	0.039	0.64
6 Next Day	0.046	0.67
7	(-) 0.016	0.14
7 Next Day	(-) 0.027	0.24

Slopes are ratio of delta pH/Ellman AChE activities.

During this past year, in parallel with the work with CHPPM, we have been working with clinical laboratories in California to assist them in developing a conversion factor between their mandated ChE assays and our reference assay, with the collaboration of the California (CA) Department of Pesticide Regulation (DPR) and the NIOSH Western Center for Agricultural Health & Safety at UC Davis. The research, recently submitted for publication (Wilson *et al.*, 2004) serves as an example of the studies we are doing with CHPPM. CA has a long-standing formal blood ChE monitoring program for mixers, loaders and applicators of pesticides.

When we found commercial clinical kits were not optimal for assaying blood ChEs, CA regulations were revised to specify use of the Ellman ChE assay or to demonstrate a conversion factor with a correlation (r^2) of 0.9 or better. When we were first enlisted by DPR to work with the clinical laboratories in 2000, only 2 of 7 participating laboratories generated an acceptable correlation for red blood cells; only 4 of 5 laboratories had an acceptable correlation for plasma ChE. Last year, when DPR restated the need to meet this requirement, we worked with several of the clinical laboratories using our bovine ghost RBC ChE as a reference. But, only 3 of 10 laboratories had acceptable correlations. Next, we provided all interested laboratories with human blood and plasma samples to perform a comparison study. Fourteen laboratories participated; 9 met the ChE criteria for whole blood, 14 for plasma and 6 for RBC. Based on such data, on July 8, 2003, DPR notified the CA Agricultural Commissioners that only 9 of the participating laboratories were approved for ChE testing. Later work resulted in acceptable RBC values for 2 of the laboratories and their approval. We continue to work with laboratories interested in being on the approved list.

Normal Range of AChE Levels: The availability of such a large number of RBC AChE samples from CHPPM provides an important opportunity to establish a normal range of human cholinesterase values better than has been done before and to convert the delta pH values to Ellman colorimetric ranges once the conversion part of Task One has been accomplished.

With the collaboration of epidemiologist-physician Dr. Stephen McCurdy, (UCD) Blood specimens from 991 Department of Defense personnel without a potential exposure to anti-cholinesterase agents were statistically analyzed. The median age was 43 years (range 18-76). 823 specimens (82.1 %) were from men. Men were on average older than women (median age 44 vs. 37 years, $p < 0.001$, Wilcoxon test). The mean \pm SD for delta pH was 0.75 ± 0.06 units. Delta-pH values were greater for men than for women (0.74 vs. 0.73, $p < 0.001$, Wilcoxon test). Multivariate linear regression analysis showed an association for delta pH with age (slope $+0.0008$ delta-pH units for each year of age, $p < 0.001$). There was a small, but statistically significant, reduction in delta pH associated with time (-0.006 delta pH units per 100 days, $p < 0.001$). A multiple regression model incorporating age, gender, and test date explained only 3.4% of the observed variance. The small magnitude of these effects and their minimal role in accounting for the observed variability suggest it appropriate to ignore such factors when evaluating delta-pH data. The next step in our project is to correlate the delta pH values with those from

the more commonly used colorimetric Ellman test. The results were reported at two meetings and are being prepared for publication (McCurdy *et al.*, 2003, 2004).

Task Two. Testing the stability and usability of a red blood cell ghost standard suitable for clinical standardizations has been carried out and the results published (Arrieta *et al.*, 2003). We have found that the activity of the preparation is too low for good detectability by the delta pH method. Plans are to prepare a standard with higher activity and retest again.

The ghost RBC standard has been useful in previous work on ChE method comparisons, such as with the Test-mate Kit (Oliveira, *et al.*, 2002), and a sample is included with each run conducted at UC Davis.

Task Three. The third goal is to conduct experiments with a specially designed Test-mate Kit with an uncorrected read out to establish the conditions for an optimum assay and construct conversion factors to harmonize its results with clinical laboratory assays. The new instrument did not become available this year. A letter from Patrick Eberly, CEO of EQM Inc. is attached as an appendix to this report. He informs us that a new, temperature regulated model is nearing the end of development but that there have been delays readying it for testing.

Task Four. The feasibility of incorporating BChE variant and PON1 polymorphisms into a screen of workers, for whom blood ChE baselines are required, using a selected set of DOD personnel will be addressed later in the project.

KEY RESEARCH ACCOMPLISHMENTS

- ◆ Correlations of RBC activity measured by the Ellman method and by the delta pH method continue and sources of variability are being investigated.
- ◆ AChE activity levels in the current samples are in agreement with the existing CHPPM data set.
- ◆ Older delta pH data are proving useful in establishing normal ranges of human RBC AChE activity.
- ◆ A bovine ghost RBC AChE standard is in use for Ellman assays and a higher activity standard will be prepared for the delta pH assays.

REPORTABLE OUTCOMES

S.A. McCurdy, J.D. Henderson, D.E. Arrieta, L.J. Lefkowitz, R.E. Reitsstetter, and B.W. Wilson. 2004. Determining a reference value for blood cholinesterase using Defense Department personnel. Abstract To Be Presented at SOT Meeting, March 2004

D. Arrieta, A. Ramirez, E. DePeters, D. Bosworth, and B.W. Wilson. 2003. Bovine red blood cell ghost cholinesterase as a monitoring standard. *Bulletin of Environmental Contamination and Toxicology* 71(3), 447-452.

B. W. Wilson, J. D. Henderson, D.E. Arrieta, and M.A. O'Malley. 2003. Assisting clinical laboratories meet requirements of cholinesterase monitoring in California. Submitted to *Int. J. of Toxicology*

S.A. McCurdy, J.D. Henderson, D.E. Arrieta, L.J. Lefkowitz, R.E. Reitstetter, and B.W. Wilson. 2003. Normal range of cholinesterase levels among US Defense Department personnel. Poster presented at NIOSH Conference, November, 2003 San Francisco, CA.

G.H. Oliveira, J.D. Henderson, and B.W. Wilson. 2002. Cholinesterase measurements with an automated kit. *American Journal of Industrial Medicine* (2002 Aug), Suppl 2: 49-53.

B.W. Wilson, J.D. Henderson, A. Ramirez, and M.A. O'Malley. 2002. Standardization of clinical cholinesterase measurements. *International Journal of Toxicology* 21(5) 385-388.

CONCLUSIONS

The first stage of establishing a conversion factor between the delta pH and Ellman assays has been completed and we are seeking to find and correct the sources of variability noted.

A statistical analysis of a set of CHPPM data has been completed, generating a distribution of AChE levels that provide the first step towards a reliable range of unexposed human RBC AChE levels useful for comparing specimens from suspected exposure episodes.

Work is proceeding on the major tasks. Unanticipated delays due to turnover of leadership at CHPPM (replacement of Capt. Reitstetter by Capt. Lefkowitz) are behind us and both scientists will continue to collaborate on the project. Capt. Lefkowitz has sent us equipment used at CHPPM. Conducting both pH and Ellman assays at UC Davis should speed up the comparisons. We await completion of the new model of the Test-Mate kit by EQM Inc.

REFERENCES

Arrieta D, Ramirez A, DePeters E, Bosworth D, Wilson BW. 2003. Bovine red blood cell ghost cholinesterase as a monitoring standard. *Bull. Environ. Contam. Toxicol.* 71(3), 447-452.

Broomfield CA, Maxwell DM, Solana RP, Castro CA, Finger AV, Lenz DE. 1991.

Protection by butyrylcholinesterase against organophosphorus poisoning in nonhuman primates. *J. Pharmacol. Exp. Ther.* 259(2): 633-638.

Ellman GL, Courtney KD, Andres V Jr., Featherstone RM. 1961. A new rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharm.* 7:88-95.

Haley RW, Billecke S, La Du BN. 1999. Association of low PON1 type Q (type A) arylesterase activity with neurologic symptom complexes in Gulf War veterans. *Toxicol. and Appl. Pharm.* 157(3):227-333.

McCurdy SA, Henderson JD, Arrieta DE, Lefkowitz LJ, Reitstetter RE, Wilson BW. 2003. Normal range of cholinesterase levels among US Defense Department personnel. Poster presented at NIOSH Conference, November, 2003 San Francisco, CA

McCurdy SA, Henderson JD, Arrieta DE, Lefkowitz LJ, Reitstetter RE, Wilson BW. 2004. Determining a reference value for blood cholinesterase using Defense Department personnel. Abstract To Be Presented at SOT Meeting, March 2004

Michel HO. 1949. An electrometric method for the determination of red blood cell and plasma cholinesterase activity. *J. Lab. Clin. Med.* 34:1564-1568.

Oliveira G.H., Henderson J.D. and Wilson B.W. 2002. Cholinesterase measurements with an automated kit. *Am. J. Indust. Med. Supplement* 2:49-53.

Shih DM, Gu L, Xia Y-R, Navab M, Li W-F, Hama S, Castellani LW, Furlong CE, Costa LG, Fogelman AM, Lusis AJ. 1998. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature (London)* 394(6690):284-287.

Wilson BW, Henderson JD, Arrieta DE, O'Malley MA. 2003. Assisting clinical laboratories meet requirements of cholinesterase monitoring in California. Submitted to *Int. J. of Toxicology*

Wilson BW. 1999. Cholinesterases. In Clinical Chemistry of Laboratory Animals. Quimby F and Loeb W (Eds). pp 430-440. Taylor and Francis Inc., Philadelphia.

APPENDICES

Figure 1. RBC Cholinesterase Assay Comparison: Sample Set 6

Figure 2. RBC Cholinesterase Assay Comparison: Sample Set 6, Next Day

Figure 3. RBC Cholinesterase Assay Comparison: Sample Set 7

Figure 4. RBC Cholinesterase Assay Comparison: Sample Set 7, Next Day

Letter from EQM, Inc.

Figure 1. RBC Cholinesterase Assay Comparison:
Sample Set 6

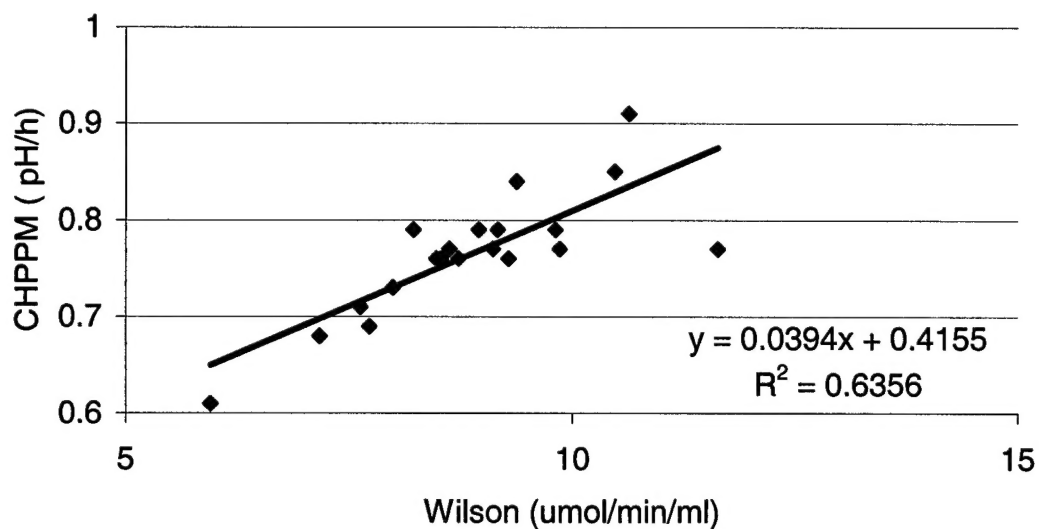


Figure 2. RBC Cholinesterase Assay Comparison:
Sample Set 6, Next Day

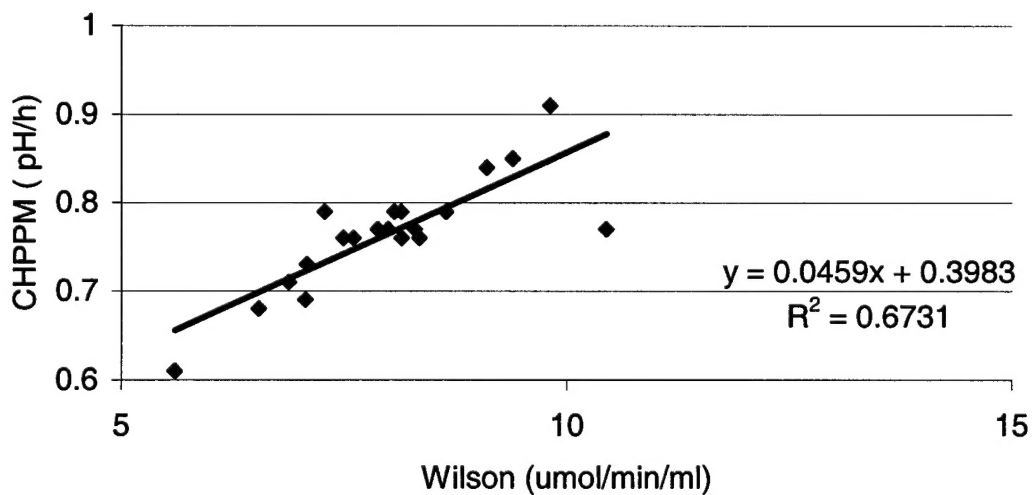


Figure 3. RBC Cholinesterase Assay Comparison:
Sample Set 7

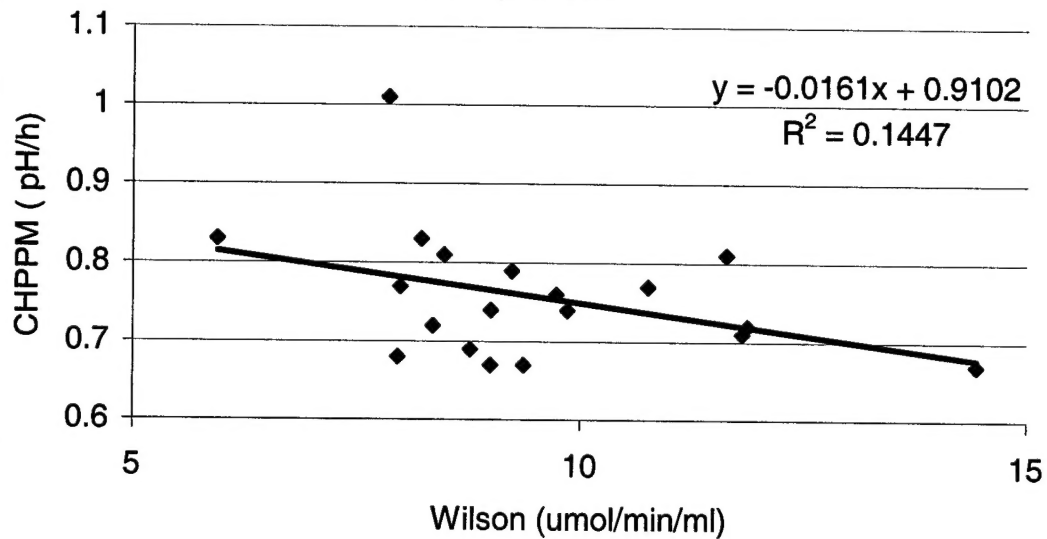
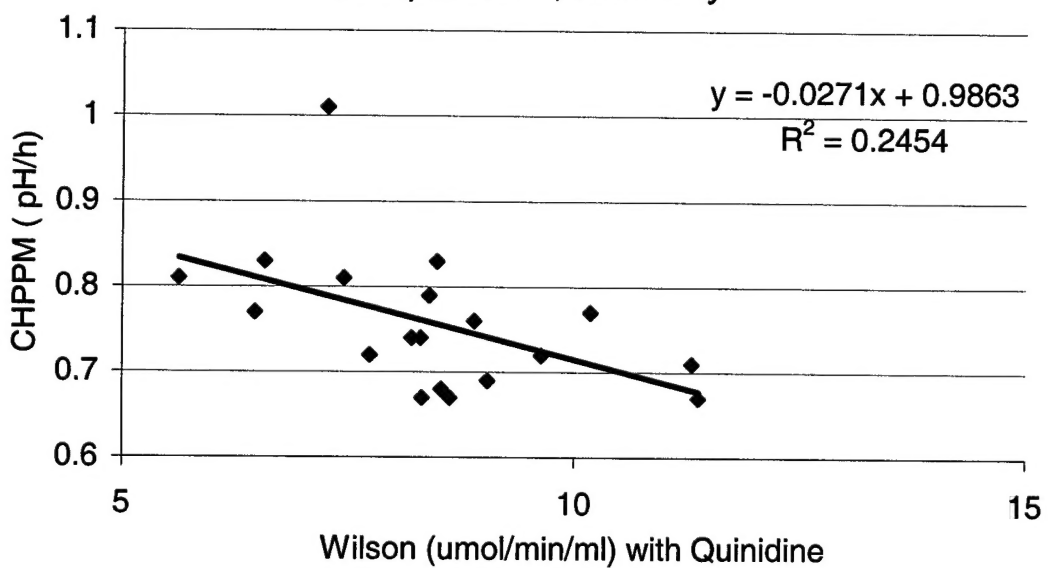


Figure 4. RBC Cholinesterase Assay Comparison:
Sample Set 7, Next Day



EQM Research, Inc.

2814 Urwiler Ave. Cincinnati, OH. 45211

Phone: (513) 661-0560 Fax: (513) 661-0567

Professor Barry W. Wilson
Department of Animal Science
Department of Environmental Toxicology
University of California
One Shields Avenue
4209 Meyer Hall
Davis, CA 95616

Phone: (530) 752-3519
Fax: (530) 752-0175
Email: bwwilson@usdavis.edu

13 October, 2003

Dear Professor Wilson:

Thank you for your continued interest in the Test-mate ChE Cholinesterase Test System. As you requested, I am writing this letter to be included with your annual report that will be submitted to USACHPPM this week. The following is a brief summary of recent research, development, and engineering accomplishments.

The new Test-mate ChE (Version D) will be offered for commercial sale in the near future. The Version D instruments are far superior to the Version C instruments that are now being sold. The new instruments will contain several design improvements that include the extension of the operational temperature range for accurate measurements of AChE, Hgb, and Q (hemoglobin corrected erythrocyte cholinesterase) to allow operation between 10°C and 50°C. As in the older Version C instruments, the Version D instruments are currently programmed to display testing results automatically adjusted to 25°C. Like the previous versions of the Test-mate ChE, the new Test-mate ChE (Version D) instruments are intended for use only with human blood. Four such new prototype instruments have been tested - two by the German Armed Forces and two by USAMRICD.

The Cholinesterase Chemistry Set (manufactured by EQM Research, Inc.), is a moderately priced collection of reagents for use in the accurate determination of AChE, BChE, and Hgb using a Molecular Devices SpectraMax microplate reader, will soon be offered for sale. This system has been extensively tested and additional testing is being scheduled. The Cholinesterase Chemistry Set presents the final results as read at the assay reaction temperature of 37°C. A detailed manuscript will be submitted to the Bioscience 2004 Review Committee.

Prior to its formal release, I am currently engaged in changing the Test-mate ChE (Version D) to display results directly comparable to The Cholinesterase Chemistry Set microplate method. This will provide consistent laboratory cholinesterase measurements and field cholinesterase measurements. Once this composite package is fully completed and more thoroughly tested, I will be glad to arrange for both a Test-mate ChE (Version D) and The Cholinesterase Chemistry Set reagents to be available to you and your laboratory personnel for evaluation.

Best Regards,



Patrick Eberly, Ph.D.
President - EQM Research, Inc